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Multiple *nodal*-Related Genes Act Coordinately in *Xenopus* Embryogenesis

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Four *nodal*-related genes (*Xnr1–4*) have been isolated in *Xenopus* to date, and we recently further identified two more, *Xnr5* and *Xnr6*. In the present functional study, we constructed cleavage mutants of *Xnr5* (*cmXnr5*) and *Xnr6* (*cmXnr6*) which were expected to act in a dominant-negative manner. Both *cmXnr5* and *cmXnr6* inhibited the activities of *Xnr5* and *Xnr6* in co-overexpression experiments. *cmXnr5* also inhibited the activity of *Xnr2, Xnr4, Xnr6, derrière,* and *BVg1*, but did not inhibit the activity of *Xnr1* or *activin*. Misexpression of *cmXnr5* led to a severe delay in initiation of gastrulation and phenotypic changes, including defects in anterior structures, which were very similar to those seen in maternal *VegT*-depleted embryos. Further, although the expression of *Xnr1, Xnr2,* and *Xnr4* was not delayed in these embryos, it was markedly reduced. Injection of *cmXnr5* had no notable effect on expression of *Xnr3, Xnr6, derrière,* or *siamois*. Several mesodermal and endodermal markers also showed delayed and decreased expression during gastrulation in *cmXnr5*-injected embryos. These results suggest that, in early *Xenopus* embryogenesis, *nodal*-related genes may heterodimerize with other TGF-β ligands, and further that one *nodal*-related gene alone is insufficient for mesendoderm formation, which may require the cooperative interaction of multiple *nodal*-related genes.

Key Words: **nodal; cleavage mutant; *Xenopus*; TGF-β superfamily; *Xnr5*; *Xnr6*.**

INTRODUCTION

In the amphibian, several maternal accumulated factors are critical for axis and germ layer formation during early development. One of these maternal factors is *VegT*, a T-box containing transcription factor, that is localized vegetally (Zhang and King, 1996). *VegT* initiates the zygotic expression of TGF-β superfamily genes and regulates many mesendodermal genes in cooperation with other maternal factors such as β-catenin (Heasman, 1997; Kimelman and Griffin, 1998, 2000). The zygotic transcripts of activin-like signal molecules of the TGF-β superfamily, such as *derrière* and the *Xenopus* nodal-related genes (*Xnrs*), are essential for mesoderm and endoderm formation (Chang and Hemmati-Brivanlou, 2000; Faure *et al.*, 2000; Kofron *et al.*, 1999). *derrière* may specifically mediate posterior induction (Sun *et al.*, 1999), while the six known *Xnrs* (Jones *et al.*, 1995; Joseph and Melton, 1997; Smith *et al.*, 1995; Takahashi *et al.*, 2000) are all strong inducers of mesendoderm, with the one exception of *Xnr3*. *Xnr3* has no apparent mesoderm or endoderm inductive abilities, and may rather be related to neural induction (Hansen *et al.*, 1997). In other vertebrates, such as zebrafish or mouse, *nodal*-related genes play essential roles in mesoderm and endoderm formation (Conlon *et al.*, 1994; Feldman *et al.*, 1998; Varlet *et al.*, 1997; Zhou *et al.*, 1993).

The activities of TGF-β superfamily molecules are regulated by many steps *in vivo*. The maturation to an active ligand consists of two processes: dimerization of precursor proteins and cleavage by subtilisin-like proprotein convertase. It has been reported that cleavage mutants of TGF-β superfamily genes, in which the consensus amino acid sequence for cleavage is changed, act as dominant-negative inhibitors (Dosch and Niehrs, 2000; Hawley *et al.*, 1995; Lopez *et al.*, 1992; Nishimatsu and Thomsen, 1998; Osada and Wright, 1999; Sun *et al.*, 1999; Wittbrodt and Rosa, 1994). The cleavage mutant protein heterodimerizes with an intact precursor protein, resulting in inhibition of mature ligand formation.

A cleavage mutant of *Xnr2* (*cmXnr2*) acted as a dominant-negative inhibitor of *Xnr1*, *Xnr2*, and *Xnr4* activity (Osada and Wright, 1999). In *Xenopus* embryos, misexpression of *cmXnr2* caused delay of dorsal lip formation and anterior

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truncations, and delayed and suppressed expression of dorsoanterior endodermal markers, suggesting that *Xnr* genes may play crucial roles in initiation of gastrulation. It was also showed that these *Xnrs* act downstream of an activin-like signaling pathway (Osada and Wright, 1999). *Xnr1*, *Xnr2*, and *Xnr4* are inducible by activin-like signaling (Clements *et al.*, 1999; Jones *et al.*, 1995; Takahashi *et al.*, 2000), whereas the recently isolated *Xnr5* and *Xnr6* are not (Takahashi *et al.*, 2000). These results suggest that *Xnr5* and *Xnr6* regulate *Xnr1*, *Xnr2*, and *Xnr4* in *Xenopus* early embryogenesis.

In the present study, we constructed cleavage mutants of *Xnr5* (*cmXnr5*) and *Xnr6* (*cmXnr6*) to examine their roles in early embryogenesis. *cmXnr5* and *cmXnr6* both inhibit the normal inductive activities of *Xnr5* and *Xnr6*, although *cmXnr5* was stronger than *cmXnr6*. *cmXnr5* also inhibited the activities of *Xnr2*, *Xnr4*, *derrière*, and *BVg1*, but not *Xnr1* or *activin*. Thus, in early *Xenopus* embryogenesis, *Xnr5* may be able to heterodimerize with *Xnr2*, *Xnr4*, *Xnr6*, *derrière*, and *Vg1*. Misexpression of *cmXnr5* leads to severe delay in initiation of gastrulation and phenotypic changes with anterior structure defects. Further, the expression of *Xnr1*, *Xnr2*, and *Xnr4*, which is thought to be highly regulated by activin-like signaling, was markedly reduced. These results support the idea that the regulatory loops of TGF-β signals are essential for mesoderm and endoderm formation.

MATERIALS AND METHODS

Construction of Cleavage Mutants

Cleavage mutants for *Xnr5* (*cmXnr5*) and *Xnr6* (*cmXnr6*) were constructed according to Hawley *et al.* (1995) and Osada and Wright (1999). The amino acid residues of the putative cleavage sites "RRHRR" (AGACGACACAGGAGG) in Xnr5 and "RRHKR" (AGAAGGCACAAGAGG) in Xnr6 were changed to "GVDGG" (GGAGTCGACGGGGGG) by overlapping PCR methods. The primers used to generate the mutation sites were: for *cmXnr5*, forward 5′-GACGGGTCGACGGGGGCAATCATTATGACCAGAAC-3′ and reverse 5′-GACGGGTCGACTCCCTTGCCTGTT-TCTCTGGTGT-3′; for *cmXnr6*, forward 5′-GACGGGT-CGACGGGGGCAACAGAAATGTACAGCAT-3′ and reverse 5′-GACGGGTCGACTCCGGTGCCAGGGAAACTGGTAC-3′. Templates for amplification were *pNRRX-Xnr5* and *pNRRX-Xnr6*. The corresponding region of *pNRRX-Xnr5* or *pNRRX-Xnr6* was replaced with the respective PCR product. *pNRRX-cmXnr5* and *pNRRX-cmXnr6* were sequenced to confirm that cloning was successful.

Embryo Manipulations and Microinjection

Xenopus laevis embryos were obtained by artificial fertilization and cultured in 10% Steinberg's solution (SS) at 20°C. Embryos were staged according to Nieuwkoop and Faber (1956). Animal cap explants were dissected at stage 9 in 100% SS and were incubated at 20°C for microscopic observation or RT-PCR.

Microinjection was performed in 100% SS containing 4% Ficoll. Capped RNAs were synthesized by using SP6 or T7 mMESSAGE mMACHINE (Ambion) with the following plasmids as templates: *pCS2-XNR1* and *pCS2-XNR2* (Sampath *et al.*, 1997); *pCS2-cmXnr2* (Osada and Wright, 1999); *pSP64TNE-Xnr4* (Joseph and Melton, 1997); *pCS2-cer-S* (Piccolo *et al.*, 1999); *pCS2-derrière* (Sun *et al.*, 1999); *pSP64T-BVg1* (Thomsen and Melton, 1993); *pSP64T-Xactivin* (Sokol *et al.*, 1991); *pNRRX-Xnr5*, *pNRRX-Xnr6*, and *pCS2-NLS-lacZ* (Takahashi *et al.*, 2000); *pNRRX-cmXnr5* and *pNRRX-cmXnr6* (described above); *pSP64T-GFP*; *Green fluorescent protein* (*GFP*) (CLONTECH) was subcloned into the *Bgl*II site of *pSP64T* (Krieg and Melton, 1984).

RT-PCR

Total RNA was prepared from embryos and explants and RT-PCR was performed as described previously (Onuma *et al.*, 1999). *Ornithine decarboxylase* (*ODC*) (Osborne *et al.*, 1991) and *Elongation factor-1 alpha* (*EF-1 α*) were used as internal controls for whole embryos and animal caps, respectively. Reverse transcriptase-negative (RT−) reactions confirmed that there was no genomic DNA contamination. All primers used have been previously described; *Xbrachyury* (*Xbra*), *goosecoid* (*gsc*), and *ODC* are in the *Xenopus* Molecular Marker Resource (http://www.cbrmed.ucalgary.ca/pvize/html/WWW/Welcome.html); *EF-1 α* and *ms-actin* (Takahashi *et al.*, 1998); *Xnr1* (Lustig *et al.*, 1996); *Xnr2*, *Xnr3*, *Xnr4*, *Xnr6*, and *derrière* (Takahashi *et al.*, 2000); *siamois* (Brannon and Kimelman, 1996); *cerberus* (Bouwmeester *et al.*, 1996); *Mixer* (Henry and Melton, 1998); *Sox17β* (Hudson *et al.*, 1997).

Whole-Mount in Situ Hybridization

Whole-mount *in situ* hybridization analysis was performed according to Harland (1991) by using albino embryos of *X. laevis*. Antisense RNA probes were synthesized with the following templates; *pXT1* for *Xbra* (Smith *et al.*, 1991), *pBluescriptSK*(−)-*goosecoid* (Cho *et al.*, 1991), *pBluescriptSK*(−)-*Xnot* (Tanegashima *et al.*, 2000), *pCS2-Mixer* (Hayata and M.A., unpublished), *pBluescriptSK*(−)-*Sox17α* (Hayata and M.A., unpublished), and *pXCG-1* (Sive *et al.*, 1989).

RESULTS

Cleavage Mutants of Xnr5 and Xnr6 Act as Dominant-Negative Inhibitors

We constructed cleavage mutants of *Xnr5* (*cmXnr5*) and *Xnr6* (*cmXnr6*) to examine their roles in early embryogenesis. The amino acid residues "RRHRR" in Xnr5 and "RRHKR" in Xnr6, which are at putative cleavage sites in these genes, were changed to "GVDGG" (Fig. 1A). We first examined whether *cmXnr5* and *cmXnr6* could inhibit secondary axis formation induced by injection of *Xnr5* and *Xnr6*, respectively (Table 1, and Fig. 1B). Both cleavage mutants showed dose-dependent inhibition of secondary axis formation induced by their corresponding genes. Microinjection of *Xnr5* (5 pg) or *Xnr6* (20 pg) RNA into the ventral marginal zone of embryos induced secondary axis formation in 60 and 59% of embryos, respectively. Injection of *cmXnr5* (50 pg) and *cmXnr6* (200 pg) RNA reduced the

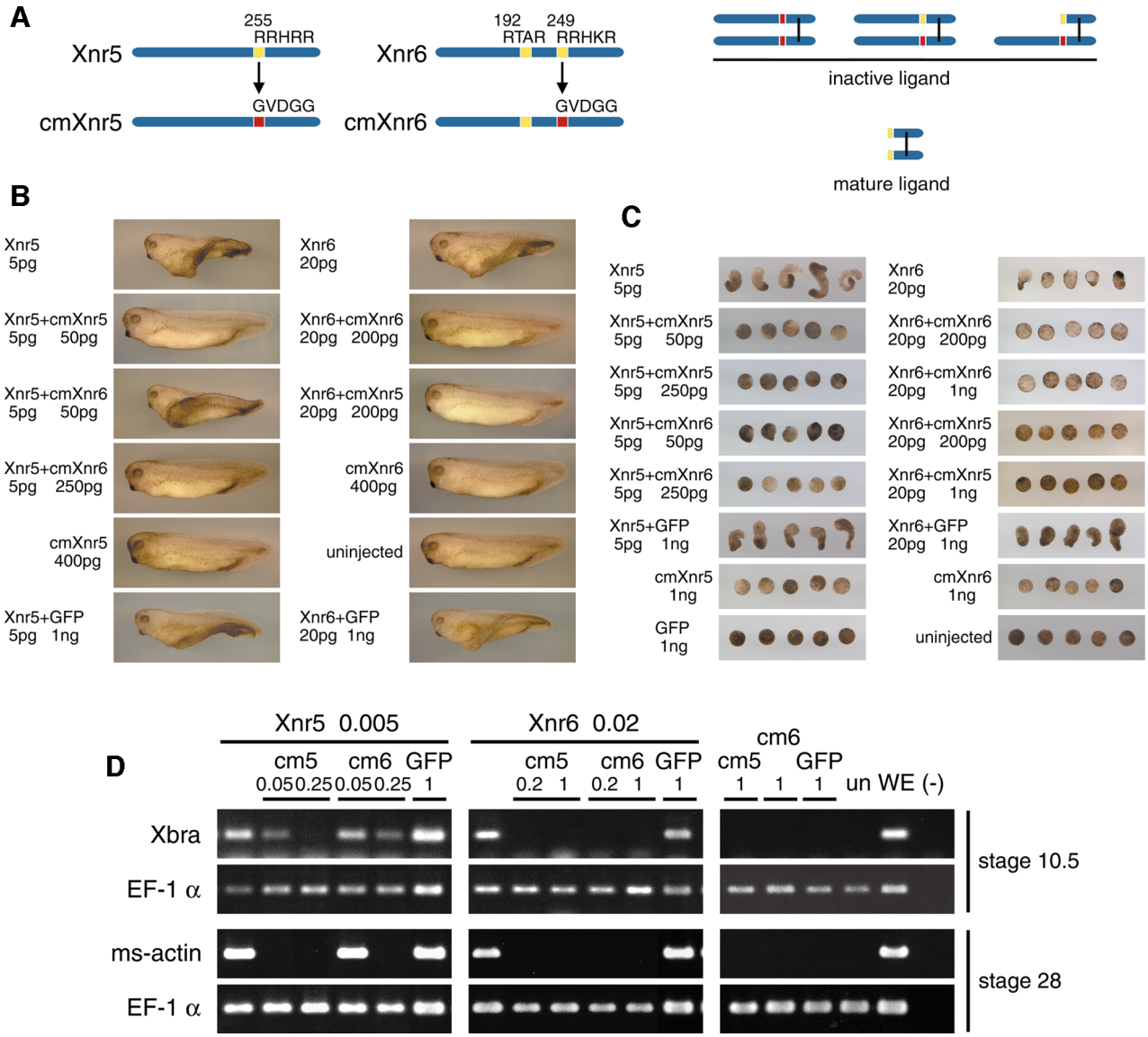

FIG. 1. *cmXnr5* and *cmXnr6* can inhibit *Xnr5* and *Xnr6* in a dominant-negative manner. (A) Model of construction and action of cleavage mutants. The putative cleavage sites "RXXR" containing sequence (yellow boxes) of Xnr5 and Xnr6, "RRHRR" and "RRHKR" were changed to "GVDGG" (red boxes). Xnr6 has two putative cleavage sites ("RTAR" and "RHKR"); the putative main cleavage site was changed. Cleavage mutant protein is not processed, and inhibits the maturation of endogenous precursor proteins by heterodimerization. Numbers on "R" residues show the number in the amino acid sequence. (B) *cmXnr5* and *cmXnr6* could prevent secondary axis formation induced by *Xnr5* and *Xnr6*. Synthesized RNAs indicated on the left side of the panel (amount per embryo) were injected into both ventral–vegetal blastomeres of eight-cell-stage embryos. *cmXnr5* RNA could inhibit secondary axis formation by both *Xnr5* and *Xnr6* at a 10-fold excess. *cmXnr6* failed to prevent secondary axis formation induced by *Xnr5* at a 10-fold excess, but was inhibitory at a 50-fold excess (see Table 1). Coinjection of 1 ng *GFP* had no effect on *Xnr5*- or *Xnr6*-injected embryos. *cmXnr5* or *cmXnr6* alone never induced secondary axis formation at the doses used. (C) Animal cap explants in stage 20. Animal caps were cut from stage-9 embryos injected with synthesized RNA into the animal pole of both blastomeres at the two-cell stage. Amounts of RNA injected (per embryo) are indicated on the left side of the panels. Animal caps elongated by injection of 5 pg of *Xnr5* or 20 pg of *Xnr6* RNA, and with coinjection of 1 ng of *GFP* RNA. Coinjection of 5 pg of *Xnr5* and 50 pg of *cmXnr6* RNAs caused some elongation of caps. Under all other conditions, explants showed no elongation. (D) RT-PCR of animal caps which were prepared the same as in (C). The amount of RNA injected (ng per embryo) is indicated on the upper side of the panel. *cmXnr5* RNA (250 pg) completely blocked the activity of 5 pg of *Xnr5* RNA. Both 50 pg of *cmXnr5* and 250 pg of *cmXnr6* RNA reduced the expression of *Xbra* (pan-mesodermal marker) and *ms-actin* (dorsal mesodermal marker) induced by *Xnr5* RNA injection, but 50 pg of *cmXnr6* RNA had little effect on the expression of these marker genes. *Xnr6* RNA (20 pg) was inhibited by injection of both *cmXnr5* and *cmXnr6*. Neither *cmXnr5* nor *cmXnr6* induced the expression of *Xbra* and *ms-actin* in animal caps. *GFP* RNA (1 ng) did not induce the expression of these mesodermal markers and had no effect on induction of these marker genes by *Xnr5* or Xnr6. EF-1 α is used as an internal control. cm5, *cmXnr5*; cm6, *cmXnr6*; un, uninjected explants; WE, whole embryo; (−), reverse transcriptase minus (RT−). RT− controls were carried out by using whole embryo samples.

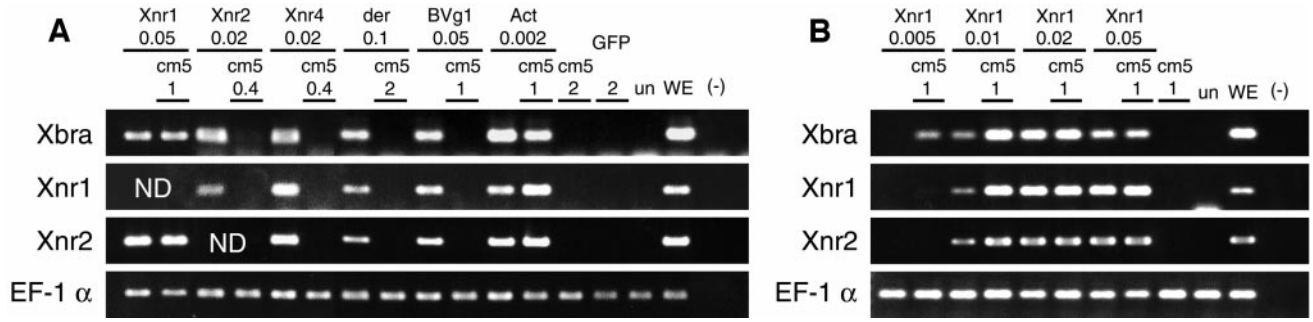

FIG. 2. Specificity of the dominant-negative inhibitory effect of *cmXnr5*. (A) Two-cell-stage embryos were injected into both blastomeres with *Xnr1* (50 pg), *Xnr2* (20 pg), *Xnr4* (20 pg), *derrière* (100 pg), or *BVg1* (50 pg) of RNA and a 20-fold excess of *cmXnr5* RNA. *Activin* (2 pg) was coinjected at a 500-fold excess of *cmXnr5* (1 ng). Animal cap ectoderm was dissected at late-blastula (stage 9) and processed for RT-PCR at stage 10.5. *cmXnr5* suppressed expression of *Xbra*, *Xnr1*, and *Xnr2* induced by *Xnr2*, *Xnr4*, *derrière*, and *BVg1*, but could not inhibit *Xnr1* or *activin*. Injection of 2 ng *cmXnr5* or *GFP* did not induce marker gene expression in animal cap explants. (B) Two-cell-stage embryos were injected into both blastomeres with increasing levels of *Xnr1* RNA (5, 10, 20, 50 pg) and a fixed amount of *cmXnr5* RNA (1 ng). Animal cap ectoderm was explanted at late blastula (stage 9) and processed for RT-PCR at stage 10.5. *cmXnr5* could not block the expression of the marker genes *Xbra*, *Xnr1*, or *Xnr2*, induced by any amount of *Xnr1* RNA. For detection of endogenous *Xnr1* transcripts, *Xnr1* primers designed against the 3′ UTR of *Xnr1* transcripts were used, which can distinguish endogenous *Xnr1* transcripts from exogenous *Xnr1* RNA. EF-1 α was used as an internal loading control for animal caps. Numbers indicate amount of RNA injected (ng). cm5, *cmXnr5*; der, *derrière*; Act, *activin*; un, uninjected explants control; WE, whole embryo positive control; (−), reverse transcriptase minus negative control. RT− controls were carried out by using whole embryo samples.

rate of secondary axis induction by both *Xnr5* (5 pg) and *Xnr6* (20 pg) to 2%, respectively. A 10-fold excess of *cmXnr5* RNA (200 pg) also inhibited *Xnr6* RNA (20 pg) activity, decreasing the rate of secondary axis formation from 59 to 1%. While a 10-fold excess of *cmXnr6* (50 pg) failed to inhibit secondary axis formation induced by 5 pg of *Xnr5*, a 50-fold excess of *cmXnr6* (250 pg) reduced secondary axis formation to 18%.

We further tested these cleavage mutants using the animal cap assay (Figs. 1C and 1D). Synthesized RNA was microinjected into the animal side of two blastomeres of two-cell-stage embryos. Animal caps were dissected from stage 9 blastulae and observed when sibling embryos reached stage 20. RT-PCR was used to detect expression of a mesodermal marker (*Xbra*) at the gastrula stage (stage 10.5) and of muscle specific-actin (*ms-actin*) at the tailbud stage (stage 28). *cmXnr5* RNA inhibited the elongation of animal caps and expression of *Xbra* and *ms-actin* induced by *Xnr5* or *Xnr6* RNA injection at a 10- and 50-fold excess. Similarly, a 10-fold excess of *cmXnr6* RNA prevented the elongation of explants and the expression of the two marker genes induced by *Xnr6*, but was insufficient to inhibit the activity of *Xnr5*. Inhibition of the activity of *Xnr5* RNA (5 pg) required coinjection of a 50-fold greater amount of *cmXnr6* (250 pg). The dominant-negative inhibitory effect of *cmXnr6* was therefore weaker than that *cmXnr5*. Injection of 1 ng of *cmXnr5* or *cmXnr6* RNA did not induce elongation of animal caps or mesodermal marker gene expression. Taken together, these results indicate that both *cmXnr5* and *cmXnr6* may act as dominant-negative inhibitors in *Xenopus* embryos.

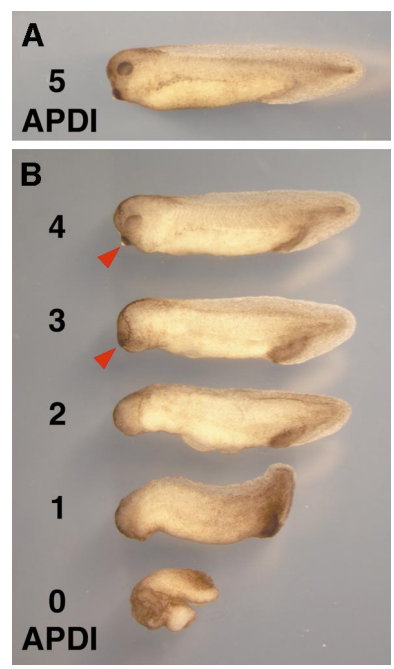

FIG. 3. Embryos injected with *cmXnr5* RNA into two dorsal blastomeres at the four-cell stage. Embryos were scored by using the APDI (anteroposterior deficiencies index). (A) Uninjected control embryo at stage 35. The normal embryo was categorized as APDI 5. (B) Embryos injected with 1 ng (0.5 ng per blastomere) of *cmXnr5*. Numbers on the left side of embryos indicate APDI. Red arrowheads indicate cement glands. Classification as APDI was as previously descrived (Osada and Wright, 1999), except that APDI2 was changed from "no eye pigment or no cement gland" to "no eye pigment and no cement gland."

TABLE 1

cmXnr5 and *cmXnr6* Can Inhibit Secondary Axis Formation Induced by *Xnr5*/*Xnr6*

RNA injected (pg)	*n*	Normal axis formation (%)	Secondary axis formation (%)	Gastrulation defected (%)	Other (%)	Dead (%)
Xnr5 (5)	166	13	60	11	13	2
+*GFP* (1000)	46	22	72		7	
+*cmXnr5* (5)	46	11	72	2	11	4
+*cmXnr5* (25)	46	63	20		15	2
+*cmXnr5* (50)	60	78	2	3	15	2
+*cmXnr5* (100)	47	85	2		13	
+*cmXnr6* (50)	165	23	56	7	13	
+*cmXnr6* (100)	48	15	50	13	23	
+*cmXnr6* (250)	93	58	18		23	1
Xnr6 (20)	144	6	59	16	18	1
+*GFP* (1000)	58	16	74		10	
+*cmXnr6* (20)	48	50	40	4	4	2
+*cmXnr6* (100)	48	83	6		10	
+*cmXnr6* (200)	64	97	2		2	
+*cmXnr6* (400)	48	90			6	4
+*cmXnr5* (200)	72	81	1		17	1
+*cmXnr5* (400)	48	81	2		17	
cmXnr5 (400)	48	77		2	19	2
cmXnr6 (400)	48	77			19	4
GFP (2000)	46	98				2

Note. Embryos were injected into two ventral blastomeres at the eight-cell stage, and scored at stage 20. All *GFP* RNA-injected embryos showed laser-induced fluorescence. Other embryos showed slightly abnormal axis formation. *n*, number of embryos.

Specificity of the Dominant-Negative Inhibitory Effect of cmXnr5

In early *Xenopus* embryogenesis, many activin-like TGF-β superfamily genes, including *activin*, *Vg1*, *Xnrs*, and *derrière*, are suspected to be involved in mesendoderm formation. One of this family of genes, *Xnr2*, was reported to be able to block *Xnr1* and *Xnr4* activities in coexpression studies (Osada and Wright, 1999). We next examined the dominant-negative inhibitory effect of *cmXnr5* on other TGF-β superfamily genes, *Xnr1*, *Xnr2*, *Xnr4*, *derrière*, *BVg1*, and *activin* (Fig. 2A). Expression of the marker genes *Xbra*, *Xnr1*, and *Xnr2*, which are induced by activin-like signals, was investigated in the animal cap assay. A 20-fold excess of *cmXnr5* RNA inhibited the induction of marker gene expression after injection of *Xnr2*, *Xnr4*, *derrière*, or *BVg1* RNA, but had no effect on those of *Xnr1*. Even a 50-fold excess of *cmXnr5* (2.5 ng) could not inhibit the activity of 50 pg *Xnr1* (data not shown). *Activin* (2 pg) was not inhibited by coinjection of 500-fold more *cmXnr5* (1 ng).

It was of interest that *cmXnr5* could block the signaling by *Xnr2*, but not that by *Xnr1*, although these two molecules have similar structures and activities. When the amount of exogenous *Xnr1* RNA was titrated down, at the lowest dose at which expression of *Xnr1* and other marker genes was seen (10 pg), *cmXnr5* still failed to inhibit expression of these genes (Fig. 2B). In fact, at this minimal dose of *Xnr1* RNA, *cmXnr5* appeared to enhance expression of both *Xnr1* and *Xbra* and *Xnr2*. The primers and template used for *Xnr1* RNA synthesis have been previously described (Lustig *et al.*, 1996; Sampath *et al.*, 1997). One of these primers was designed against the *Xnr1* 3′ UTR, which was not present in the template. Thus, only endogenous *Xnr1* transcripts were detected in the *Xnr1*-injected embryos. Taken together, these data indicate that *cmXnr5* selectively inhibited *Xnr2*, *Xnr4*, *Xnr5*, *Xnr6*, *derrière*, and *Vg1*, making it a useful tool for loss-of-function analyses of these TGF-β superfamily genes and analysis of the roles of *Xnr1* and *activin* on *Xenopus* embryogenesis.

Misexpression of cmXnr5 Leads to Severe Delay of Gastrulation and Anterior Defects

To examine the effects of *cmXnr5* on early embryos, we microinjected *cmXnr5* RNA into two dorsal or ventral blastomeres of four-cell-stage embryos. Embryos injected with *cmXnr5* showed significant anterior defects, so we categorized embryos by the anteroposterior deficiencies index (APDI) at the tadpole stage (Osada and Wright, 1999; Wallingford *et al.*, 1997) (Fig. 3). Dorsal injection of 1 ng *cmXnr5* RNA induced various levels of anterior defects (Fig. 3B), and these effects were dose-dependent (Table 2). Ventral injection of *cmXnr5* RNA caused anterior defects similar to those seen with dorsal injection. In a previous study (Osada and Wright, 1999), *cmXnr2* antagonized Xnrs signaling and caused anterior truncation of embryos. We therefore compared the effects of *cmXnr2* and *cmXnr5* (Table 2). The phenotypes induced by these two different

TABLE 2
Effect of RNA Concentration and Site of Injection on Phenotype of Embryos

RNA injected (pg)			*n*	Anteroposterior deficiencies index (APDI) (%) 5	4	3	2	1	0	Exogastrulated (%)	Average APDI	Other (%)	Dead (%)
cmXnr5	250	DMZ	47	30	38	17	4				4.0		11
		VMZ	47	53	21	11	2	2			4.4	2	9
	500	DMZ	45		24	38	22	2			3.0	7	7
		VMZ	46	33	35	24		2			4.0		7
	1000	DMZ	43		12	53	14	5	7		2.6	2	7
		VMZ	47		43	53			4		3.3		
	2000	DMZ	71			76	3	14	1	1	2.6	1	3
		VMZ	69		12	72		6	1	6	2.8	3	
cmXnr2	50	DMZ	47	19	60	11	4	4			3.9	2	
		VMZ	48	60	38	2					4.6		
	100	DMZ	48			27	40	23	4	4	1.9		2
		VMZ	44	14	48	27	5	5			3.6		2
	250	DMZ	47					2	30	60	0.0		9
		VMZ	45				2	7	27	51	0.1		13
	500	DMZ	47						13	77	0.0		11
		VMZ	48						10	75	0.0		15
	1000	DMZ	52					4		50	0.1		46
		VMZ	44						5	64	0.0	2	30
GFP	2000	DMZ	48	92					2		4.9	6	
		VMZ	47	83							5.0	15	2
Uninjected			65	97							5.0	3	

Note. Embryos were injected into two dorsal or ventral blastomeres at the four-cell stage. All *GFP* RNA-injected embryos showed laser-induced fluorescence. Embryos were scored by the anteroposterior deficiencies index (APDI) at stage 35 (see Fig. 3) and calculation of the average APDI (exogastrulae were regarded as APDI 0, and nonscorable embryos were eliminated) was performed according to Osada and Wright (1999). DMZ, dorsal marginal zone; VMZ, ventral marginal zone; *n*, number of embryos.

cleavage mutants were nearly identical, but the effects of *cmXnr2* were much stronger than those of *cmXnr5*. Similar anterior defects were also caused by injection of 2 ng of *cmXnr6*, but at a much lower rate than with the same amount of *cmXnr5* RNA. The average APDI was 3.1 in embryos injected dorsally and 3.8 in those injected ventrally (data not shown).

Xnr1, *Xnr2*, *Xnr4*, *Xnr5*, *Xnr6*, *derrière*, and *Vg1* are widely expressed in the vegetal hemisphere, the endodermal germ layer region, at blastula stage (Agius *et al.*, 2000; Jones *et al.*, 1995; Sun *et al.*, 1999; Takahashi *et al.*, 2000; Weeks and Melton, 1987). We microinjected *cmXnr5* concurrently into four vegetal blastomeres (Fig. 4, and Table 3). Embryos injected with *cmXnr5* showed apparent delays in the start of gastrulation compared with control embryos (Figs. 4A–4D). In addition, gastrulation and neural plate formation were abnormal; gastrulation was incomplete, leaving a large portion of the blastocoel, and then the neural plate formed at the marginal or vegetal region which was unpigmented. When the embryos reached the tailbud stage, the injected embryos had a pigmented ventral surface but were unpigmented on the dorsal region (Fig. 4F). At the tadpole stage (Fig. 4H), the embryos had a shortened body axis with a darkly pigmented ventral and unpigmented dorsal surface, and no distinguishable anterior structures except for a cement gland. Cement gland formation was confirmed by *in situ* hybridization analysis using the cement gland-specific marker *XCG-1* (Sive and Bradley, 1996; Sive *et al.*, 1989) (Fig. 4J). These phenotypes were identical or very similar to the maternal *VegT*-depleted embryos generated by injection of antisense oligodeoxynucleotides (Zhang *et al.*, 1998). Likewise, injection of *cerberus-short* (*cer-S*), which is a specific antagonist of all *Xnrs* except *Xnr3* (Agius *et al.*, 2000; Piccolo *et al.*, 1999; Takahashi *et al.*, 2000), gave rise to a strikingly similar phenotype (Fig. 4I). These results indicated that one or more of the genes that are inhibited by *cmXnr5* (*Xnr2*, *Xnr4*, *Xnr5*, *Xnr6*, *derrière*, or *Vg1*) play essential roles in early *Xenopus* embryogenesis, and that inhibition of these signaling events causes phenotypic changes resembling those induced by *cer-S* injection or *VegT* depletion.

cmXnr5 Inhibits Mesodermal and Endodermal Formation

Previous studies showed that *cmXnr2* affects the expression of genes related to mesoderm and endoderm specification (Osada and Wright, 1999; Xanthos *et al.*, 2001). Head organizer endoderm markers in particular are prominently suppressed and mesodermal marker expression is delayed

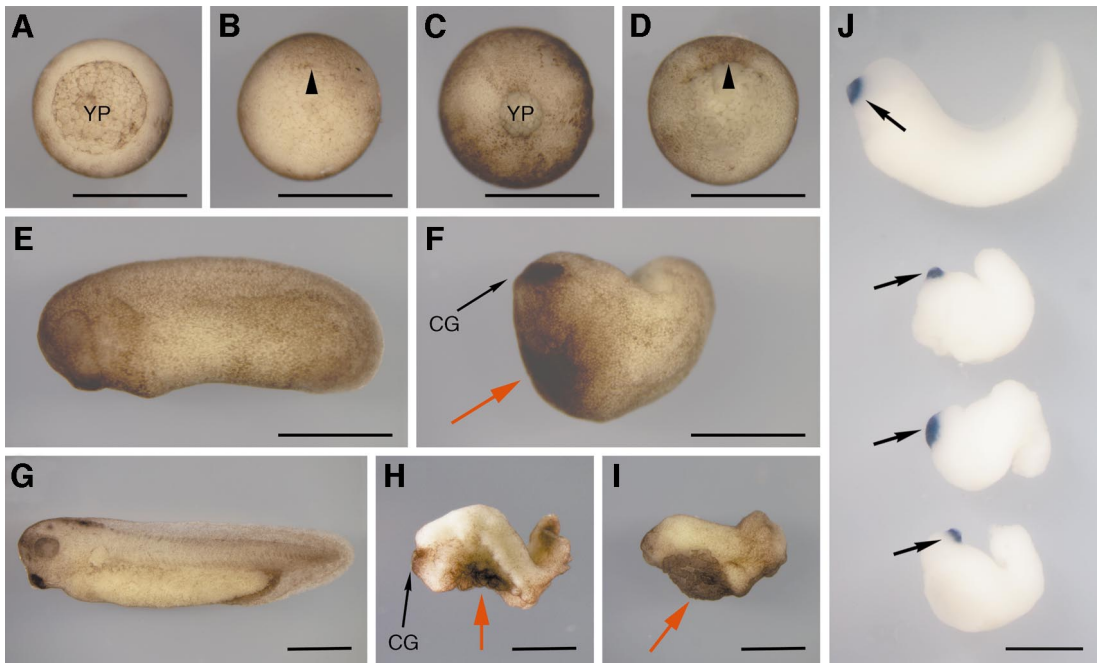

FIG. 4. *cmXnr5* causes severe delay of gastrulation and abnormal axis formation. Embryos were injected with RNA into four vegetal blastomeres at the eight-cell stage. (A) Uninjected control embryo at stage 11. (B) Embryo injected with 4 ng (1 ng per blastomere) of *cmXnr5* RNA at stage 11 had delayed dorsal lip formation and was slightly pigmented on the invagination (arrowhead). (C) Uninjected control embryo at stage 12. (D) Embryo injected with 4 ng (1 ng per blastomere) of *cmXnr5* at stage 12. The dorsal lip was formed (arrowhead). (E) Uninjected control embryo at stage 25. (F) Embryo injected with 4 ng (1 ng per blastomere) of *cmXnr5* at stage 25. (G) Embryo injected with 4 ng (1 ng per blastomere) of *GFP* at stage 35, showed an altered phenotype. (H) Embryo injected with 2 ng (0.5 ng per blastomere) of *cmXnr5* at stage 35, had a shortened body axis with darkly pigmented ventral and unpigmented dorsal surface, and no distinguishable anterior structures except a cement gland. (I) Embryo injected with 1 ng (0.25 ng per blastomere) of *cer-S* showed VegT-depleted-like phenotypes (see text). Red arrows indicate the pigmented region derived from the blastocoel in (F), (H), and (I). (J) An uninjected control embryo (top) and 4 ng (1 ng per blastomere) of *cmXnr5*-injected embryos (bottom three) at stage 35. Arrow indicates a cement gland expressing a cement gland-specific marker *XCG-1*. (A–D) Vegetal view; (E–J) lateral view. YP, yolk plug; CG, cement gland. Scale bars indicate 1 mm.

(Osada and Wright, 1999). *cmXnr5* caused similar anterior defects to *cmXnr2* (Fig. 3, and Table 2), so we investigated the effects of *cmXnr5* on expression of several mesendodermal markers and TGF-β superfamily members (Fig. 5A). From blastula to gastrula stage, injection of *cmXnr5* RNA caused distinctly delayed and reduced expression of meso-

TABLE 3

Phenotypes of *cmXnr5*-Injected Embryos

RNA injected (ng)		*n*	*VegT*-depleted-like (%)	Anterior defected (%)	Exogastrulated (%)	Normal (%)	Other (%)	Dead (%)
cmXnr5	2	71	49	22		6	10	13
	4	40	65	15	8	5		8
cmXnr2	0.5	63		25	37		2	37
	1	57		14	42		2	42
	2	45		16	51			33
Cer-S	1	45	87		4		9	
GFP	4	70		4		86	4	6
Uninjected		87				99	1	

Note. Embryos were injected into all four vegetal blastomeres at the eight-cell stage, and scored at stage 35 for phenotypes as described in the text. All *GFP* RNA-injected embryos showed laser-induced fluorescence. Embryos with anterior defects showed an APDI of 0-4. *n*, number of embryos.

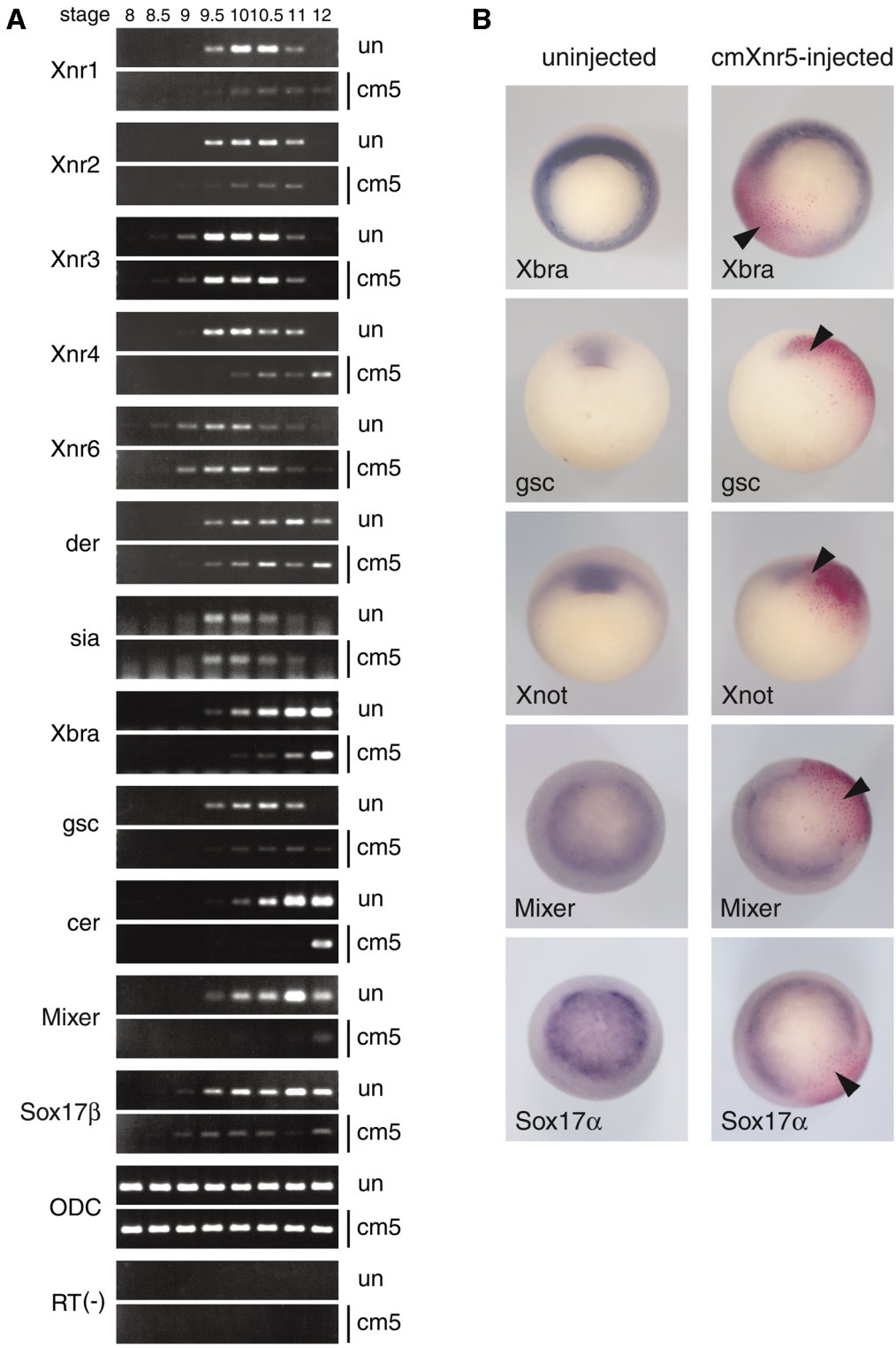

FIG. 5. Inhibition of early mesodermal and endodermal gene expression in embryos by *cmXnr5*. (A) RT-PCR of whole embryos injected with 4 ng (1 ng per blastomere) of *cmXnr5* into four vegetal blastomeres at the eight-cell stage. Embryos were sampled at the indicated stage (stages 8–12). *ODC* was used as an internal control. un, uninjected embryos; cm5, *cmXnr5*-injected embryos; der, *derrière*; sia, *siamois*; gsc, *goosecoid*; cer, *cerberus*; RT(−), reverse transcriptase minus. RT− controls were carried out by using an *ODC* primer. (B) Embryos were coinjected with 1 ng *cmXnr5* and 250 pg of *NLS-lacZ* RNAs into one vegetal blastomere at the eight-cell stage, and were stained with the RedGal substrate and then analyzed by whole-mount *in situ* hybridization at the gastrula stage (stage 11). Uninjected control embryos showed normal expression of *Xbra*, *Xnot*, *goosecoid*, *Mixer*, and *Sox17α* (purple staining). *cmXnr5* and *NLS-lacZ* RNA coinjected embryos displayed suppressed marker gene expression (arrowhead) within or adjacent to the region of RedGal labeling (red). All embryos were in vegetal view. uninjected, uninjected embryos; cmXnr5-injected, *cmXnr5* and *NLS-lacZ* RNAs coinjected embryos.

dermal and endodermal genes, such as *Xbra* (Smith *et al.*, 1991), *goosecoid* (Cho *et al.*, 1991), *cerberus* (Bouwmeester *et al.*, 1996), *Mixer* (Henry and Melton, 1998), and *Sox17β* (Hudson *et al.*, 1997). There was a significant delay in the expression of *Xbra* and *cerberus*, and a remarkable decrease in the expression of *goosecoid*, *Mixer*, and *Sox17β*, compared with uninjected control embryos. The expression of *Xnr1*, *Xnr2*, and *Xnr4* was not delayed, but was dramatically reduced. *cmXnr5* had no effect on expression of *Xnr3*, *Xnr6*, *derrière*, or *siamois* (Lemaire *et al.*, 1995). In addition, whole-mount *in situ* hybridization analysis and lineage-tracer study showed that the expression of mesodermal genes (*Xbra*, *goosecoid*, and *Xnot*; von Dassow *et al.*, 1993) was inhibited in a region adjacent to the derivative of a blastomere coinjected with *cmXnr5* RNA and a lineage-tracer *NLS-lacZ* RNA (Fig. 5B). The expression of two suppressed endodermal genes (*Mixer* and *Sox17α*; Hudson *et al.*, 1997) was also detected within a region of RedGal labeling (Fig. 5B). These results indicate that *cmXnr5* exerts its effects by inhibition of Xnrs, derrière, and/or Vg1 signaling and inhibits both early mesoderm and endoderm formation.

Both Xnr5 and Xnr6 Are Inhibited by cmXnr2

cmXnr2-injected embryos did not show the *VegT*-depleted-like phenotype at any dose (Table 3). Low-dose *cmXnr2* caused anterior defects and high doses caused exogastrulation. *cmXnr2* has been reported to be a specific dominant-negative inhibitor of *Xnr1*, *Xnr2*, and *Xnr4* (Osada and Wright, 1999). Thus, we predicted that *cmXnr2* cannot inhibit *Xnr5* and *Xnr6* activities, and it was confirmed by analysis of secondary axis formation and animal cap assay. However, *cmXnr2* prevented the secondary axis formation induced by both *Xnr5* and *Xnr6* (Fig. 6A), and inhibited the *Xbra* expression in explants induced by *Xnr5* and *Xnr6* as well as by *Xnr2* at a 10-fold excess (Fig. 6B). These data suggest that *cmXnr2*, like *cer-S*, is a specific inhibitor of all *Xnrs* except *Xnr3*.

DISCUSSION

Interaction of Xnrs and Other TGF-β Superfamily Genes in Early Xenopus Embryogenesis

Previous studies have shown that TGF-β superfamily proteins require cleavage by subtilisin-like proprotein convertases to become active. The consensus amino acid sequence for the cleavage of TGF-β superfamily members is "RXXR." In this study, *cmXnr5* and *cmXnr6* had similar dominant-negative inhibitory activities and no mesoderm-inducing activities, but the inhibitory activity of *cmXnr6* was much weaker than that of *cmXnr5*. Xnr6, but not Xnr5, has an additional putative cleavage site in the proregion, which may have influenced the inhibitory activity of *cmXnr6*. Cleavage mutants of *Xnr2* showed strong dominant-negative inhibitory activity, whereas cleavage mutants of *Xnr1* and *Xnr4* did not inhibit any *Xnrs* and retained mesoderm-inducing activities (Osada and Wright, 1999). Both Xnr2 and Xnr1 have additional putative cleavage sites to the mutated site (Jones *et al.*, 1995). However, it was puzzling that an *Xnr4* cleavage mutant had no inhibitory activity, although Xnr4 has only one putative cleavage site (Joseph and Melton, 1997). The activity of a cleavage mutant construct should be closely related to the actual processing site of the intact precursor protein. Further analysis of posttranslational regulation of protein maturation is required to clarify this problem.

In this study, we showed that *cmXnr5* could prevent mesoderm induction by *Xnr2*, *Xnr4*, *Xnr5*, and *Xnr6*, and that *cmXnr2* could also inhibit the effects of *Xnr5* and *Xnr6*. These *Xnrs* are coexpressed in a wide range of vegetal regions from dorsal to ventral at late blastula stage (Agius *et al.*, 2000; Jones *et al.*, 1995; Takahashi *et al.*, 2000), and therefore may function in heterodimers with other *Xnrs*. However, the mechanism of selecting a counterpart for dimerization may be complicated and may not be simply explained by sequence similarity. *cmXnr2* specifically blocked mesoderm induction by *Xnr1*, *Xnr2*, and *Xnr4* (Osada and Wright, 1999), while *cmXnr5* could not prevent *Xnr1* activity. It is very interesting that *Xnr1* is inhibited by *cmXnr2* not but *cmXnr5*, though the primary structure similarity between *Xnr1* and *Xnr5* is almost equal to that between *Xnr1* and *Xnr2* (Takahashi *et al.*, 2000).

cmXnr5 could also inhibit the activities of other TGF-β superfamily genes such as *derrière* and *BVg1*. *derrière* is expressed in vegetal to marginal zone cells and maternal-derived *Vg1* RNA is present in the widely vegetal hemisphere at blastula stage (Sun *et al.*, 1999; Weeks and Melton, 1987). These results suggest that *Xnrs* can heterodimerize with other TGF-β superfamily members such as *derrière* and *Vg1*. Previous studies have shown that dominant-negative cleavage mutants can only act on other members of the same subgroup of genes. For example, *BMP* cleavage mutants can heterodimerize with other members of BMPs and block their activity (Hawley *et al.*, 1995; Nishimatsu and Thomsen, 1998). *cmXnr2* specifically blocks mesoderm induction by all *Xnrs* except *Xnr3* (Osada and Wright, 1999). Conversely, many studies using dominant-negative cleavage mutants have shown that these mutants have very little influence on activities of members of different subgroups. *cmXnr2* cannot inhibit *BVg1* and *activin* (Osada and Wright, 1999). *cm-derrière* severely attenuates the activity of *derrière* and slightly interferes with *Xnrs* but does not inhibit *BVg1* or *activin* activity (Sun *et al.*, 1999), and *cm-activin* specifically blocks *activin* (Hawley *et al.*, 1995; Osada and Wright, 1999). In addition, a dominant-negative *Vg1* mutant did not block *activin* or *Xnrs* (Joseph and Melton, 1998). Thus, this is the one of few studies which demonstrates that a dominant-negative mutant has clearly cross-reacted with members of other TGF-β family subgroups.

Differences between Cleavage Mutants of Xenopus nodal-Related Genes

Five of six *Xenopus* nodal-related genes have very similar mesoderm- and endoderm-inducing activities, and all share high degrees of sequence homology. To date, only the dominant-negative *Xnrs* cleavage mutant *cmXnr2* has been reported (Osada and Wright, 1999). In the present study, cleavage mutants of *Xnr5* and *Xnr6* were both shown to act as dominant-negative inhibitors with similar effects, although *cmXnr5* had stronger inhibitory actions. Comparison of the effects of *cmXnr5* and *cmXnr2* shows a number of differences in their activities. They have different specificites; *cmXnr2* can block the signaling of *Xnr1*, *-2*, *-4*, *-5*, and *-6*, but not *BVg1* or *activin* (Osada and Wright, 1999), whereas *cmXnr5* can inhibit the signaling of *Xnr2*, *-4*, *-5*, *-6*, *derrière*, and *BVg1*, but not *Xnr1* or *activin*. Secondly, while both embryos injected with *cmXnr2* and *cmXnr5* into two dorsal or ventral blastomeres at the four-cell stage showed significant and similar anterior defects, the effects of *cmXnr2* were much stronger than those of *cmXnr5*. In addition, *cmXnr2* tended to cause exogastrulation, while *cmXnr5*-injected embryos had a tendency to form the cement gland. These differences may reflect the differential inhibitory effects of these two cleavage mutants. Thirdly, there were marked phenotypic differences between embryos injected with either cleavage mutant into four vegetal blastomeres at the eight-cell stage. Although both *cmXnr2* and *cmXnr5* caused apparent delays in the initiation of gastrulation, at later stage, *cmXnr5*-injected embryos showed a *VegT*-depleted-like phenotype, which was never seen with *cmXnr2*-injected embryos. *cmXnr2* caused anterior defects at low doses and exogastrulation at high doses after injection into only two dorsal blastomeres at the four-cell stage (Osada and Wright, 1999), or into all vegetal blastomeres at the eight-cell stage (Table 3). Surprisingly, although both *cmXnr2* and *cer-S* equally inhibit all *Xnrs* except *Xnr3*, *cer-S* can efficiently induce a *VegT*-depleted-like phenotype like *cmXnr5* (Table 3). Further studies are needed to resolve these differences. A fourth difference is the expression of mesodermal and endodermal genes in injected embryos. Previous studies of *cmXnr2* showed that dorsoanterior endodermal gene expression is more highly influenced than mesodermal gene expression from blastula to gastrula (Osada and Wright, 1999). On the other hand, injection of *cmXnr5* caused severe delay and suppression of mesodermal gene expression as well as endodermal gene expression. Both experiments were carried out under slightly different conditions. *cmXnr5* was injectioned into all vegetal blastomeres at the eight-cell stage, whereas *cmXnr2* was injectioned into all blastomeres equatorially at the four-cell stage. However, the distinct effects of *cmXnr5* support the idea that the activities of the two cleavage mutants are qualitatively different. The result is different from the expression of genes in *cmXnr5*-injected embryos into all vegetal blastomeres at the eight-cell stage. Injection of *cmXnr5* caused severe delay and suppression of mesodermal gene expression as well as endodermal gene expression. These results support the idea that the effects of the two cleavage mutants are qualitatively different.

Multiple TGF-β Superfamily Gene Signaling in Mesendoderm Formation

When *cmXnr5* RNA was injected into embryos, the signaling of *Xnr2*, *Xnr4*, *Xnr5*, *Xnr6*, *derrière*, and *Vg1* was blocked, but *Xnr1* and *activin* were not inhibited. In these embryos, the initiation of expression of *Xnr1*, *Xnr2*, and *Xnr4* was not delayed but the level of expression was markedly reduced. This may be explained by the suggestion that these *Xnrs* are directly induced by *VegT* or other maternal factors (Kofron *et al.*, 1999), and then their expression is up-regulated by activin-like signaling and maintained by a positive feedback system (Agius *et al.*, 2000; Hyde and Old, 2000; Osada *et al.*, 2000; Yasuo and Lemaire, 1999). In *cmXnr5*-injected embryos, the reduced expression of these three *Xnrs* suggested that the residual *Xnr1* was insufficient to sustain normal levels of expression of these *Xnrs*. Expression of *Xnr3*, *Xnr6*, and *siamois* was unaffected by *cmXnr5* injection. It has been suggested that induction of *Xnr3* and *siamois* is strongly dependent on β-catenin signaling (Brannon and Kimelman, 1996; Carnac *et al.*, 1996; Heasman *et al.*, 2000; Smith *et al.*, 1995), and that *Xnr6* is directly regulated by *VegT*/β-catenin or other maternal factors (Takahashi *et al.*, 2000). Our results support the idea that the endogenous expression of these genes does not require activin-like signaling. Furthermore, expression of *derrière* was not markedly affected by the *Xnr5* dominant-negative inhibitor. *derrière* is induced in animal caps by activin-like signaling (Sun *et al.*, 1999), but its expression is not affected by dominant-negative *typeII activin-receptor* injection or vegetal cell disaggregation (Agius *et al.*, 2000; Yasuo and Lemaire, 1999), suggesting that *derrière* is regulated by a cell-autonomous mechanism rather than by activin-like signaling. Many mesodermal and endodermal genes have been shown to be highly regulated by zygotic activin-like signaling (Agius *et al.*, 2000; Kofron *et al.*, 1999; Osada and Wright, 1999; Xanthos *et al.*, 2001). Injection of *cmXnr5* also caused severe defects in the expression of mesodermal and endodermal genes, such as prominent delay in the expression of *Xbra* and *cerberus* and marked suppression of the expression of *goosecoid*, *Mixer*, and *Sox17β*.

Previous studies have demonstrated that *cer-S* is an antagonist of *Xnrs* and causes inhibition of mesoderm formation in the presence of endogenous *derrière*, *activin*, and *Vg1* mRNAs in injected embryos (Agius *et al.*, 2000; Piccolo *et al.*, 1999; Takahashi *et al.*, 2000), and *cer-S*-injected embryos closely resembled the *VegT*-depleted phenotypes (Fig. 4I). It is indicated that *Xnrs* activities are essential for mesoderm and endoderm formation. On the other hand, although *cmXnr5* could not inhibit the activities of *Xnr1*, embryos injected with *cmXnr5* were similar to the *VegT*-depleted phenotypes and the *cer-S*-injected embryos, and had the abnormal expression of mesendodarmal genes. In those embryos, the autonomously induced *Xnr1* was insufficient to induce the normal accumulation of *Xnr1*, although *Xnr1* is directly regulated by maternal *VegT* and is autoactivated by itself signaling (Agius *et al.*, 2000; Hyde and Old, 2000; Kofron *et al.*, 1999; Osada *et al.*, 2000; Yasuo and Le-

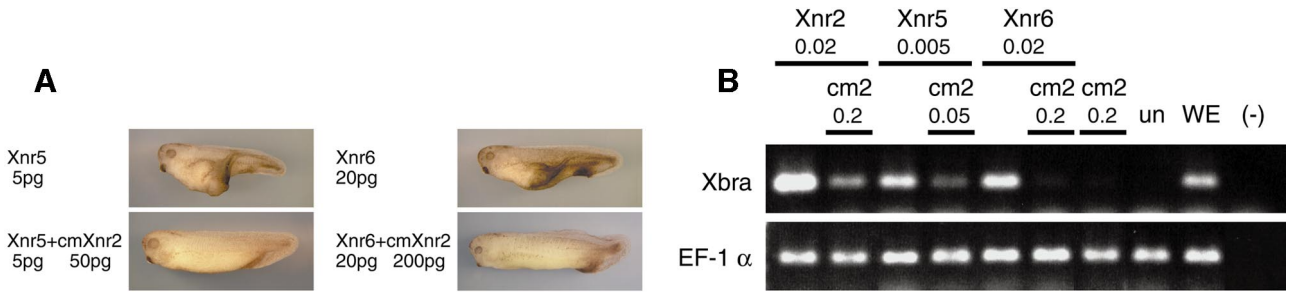

FIG. 6. *cmXnr2* can inhibit *Xnr5* and *Xnr6* in a dominant-negative manner. (A) A 10-fold excess of *cmXnr2* prevented secondary axis formation induced by both *Xnr5* and *Xnr6*. RNAs indicated on the left side were injected into both ventral–vegetal blastmeres of eight-cell-stage embryos (per embryo). (B) Animal caps were cut from late-blastulae that had been injected into the animal poles of two-cell-stage embryos for RT-PCR. *cmXnr2* RNA inhibited *Xbra* expression induced by *Xnr2*, *Xnr5*, and *Xnr6* RNA at a 10-fold excess. EF-1 α was used as an internal control. Numbers indicate amount of injected RNA per embryo (ng). cm2, *cmXnr2*; un, uninjected explants; WE, whole embryo; (−), reverse transcriptase minus (RT−). RT− controls were carried out by using whole embryo samples.

maire, 1999). These data suggest that the endogenous *Xnr1* expression needs other *Xnrs* signaling. In conclusion, cooperation and regulatory loops of multiple *nodal*-related genes are essential for mesendoderm formation in early *Xenopus* embryos.

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